

# Metallothionein: Structure/Antigenicity and Detection/Quantitation in Normal Physiological Fluids

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Recent experiments in the application of radioimmunoassay (RIA) in the detection and quantitation of metallothionein (MT) in human sera and urines demonstrate that it is possible to extend the lower limit of practical quantitation from the previous limit of 50–100 pg to 1 pg. RIA of normal sera indicates that the typical range of concentrations of MT is from less than 0.01 ng/mL to about 1 ng/mL, and that concentrations above 2 ng/mL should be considered abnormal. The typical range for normal urines is from less than 1 ng/mL to 10 ng/mL; concentrations above 10 ng/mL should be considered abnormal. A complementary assay, the enzyme-linked immunosorbent assay (ELISA), is under development. The ELISA is a competitive binding assay, detection and quantitation of MT being either by colorimetric or fluorimetric methods. The present useful range for MT quantitation in the ELISA is from about 50–50000 pg (fluorimetric) or 500–5000 pg (colorimetric).

Recent experiments using the RIA have identified the principal antigenic determinants of vertebrate MTs as involving the immediate amino terminal residues (–MDPNC–) and the segment including residues 20–25 (–KCKECK– in human MT). Theoretical predictions of secondary structure based on hydrophilicity and sequence analysis indicate that the conformational profile is dominated by tetrapeptide candidates for beta turns (reverse turns) with 2–3 hexapeptide sequences being candidates for helical conformation and 4–5 short sequences (3–5 residues) being candidates for beta chain conformation. The helical candidates are predicted to be unstable and the analysis favors reverse turns for both determinants of vertebrate MT and a sequestered location for the joining region between clusters A and B.

## Introduction

Since the development of the double-antibody radioimmunoassay (RIA) for metallothionein (MT) in my laboratory (1–3) in 1977–1979, it has been applied in numerous studies of that protein, including application to the determination of MT content in human sera and urine prior to and after exposure to cadmium (4–7), in the serum of rats treated with cadmium and zinc (8), to the identification of certain metal-binding proteins as MTs (9,10) and to the determination of the degree to which certain invertebrate metal-binding proteins and candidate vertebrate MTs cross-react with known vertebrate MTs. These studies stimulate interest in more firmly defining the typical

range of concentrations of MT in physiological fluids of normal human subjects and in similarly defining more firmly the precise nature of the reaction occurring in the RIA. Therefore, we have made regular modifications and improvements to the protocols to permit determinations of MT content in body fluids at progressively decreasing MT concentrations, and we have performed studies aimed at improving our knowledge of the processes involved in the *in vitro* reactions between MT as antigen and the specific anti-MT antibody. The former effort has gradually led to a capability to extend the lower limit of detection of MT in body fluids from 1–2 ng/mL to 0.02 ng/mL. As a complement to the RIA we have initiated development of an enzyme-linked immunosorbent assay (ELISA). The effort to improve understanding of the details of the antigen-antibody reaction has led to the identification of the principal antigenic determinants of vertebrate MTs

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(11) and concomitantly to useful information concerning the secondary, and perhaps some aspects of the tertiary, structure of the protein. We discuss these recent improvements and applications below.

## Metallothionein in Normal Serum and Urine

### Detection/Quantitation by Double-Antibody RIA

The protocols for the double-antibody RIA have been previously described (1-3,12); we comment briefly on them here. Conventionally, the antigen (Ag), rat MT isolated from kidney or liver tissue, is injected into rabbits to stimulate the production of primary antibodies (Ab) which later are used in the *in vitro* RIA to recognize and to bind <sup>125</sup>I-labeled MT or unlabeled MT in competitive binding assays. Known amounts of labeled and unlabeled MT are used in conjunction with known amounts of primary Ab and of a secondary Ab (goat anti-rabbit IgG) to produce a standard curve that is then used to quantitate samples containing unknown amounts of MT. The second Ab is used to precipitate Ag-Ab complexes, avoiding the complications which accompany the use of chemical reagents rather than a second Ab for this purpose. In our initial work we developed linear regressions relating the fraction (Y) of bound labeled MT to the log of the concentration (X) of unlabeled MT. The practical limit was about 2 ng/mL, this in part a consequence of the sigmoid character of the response. Subsequently, we developed inverse variance weighted logit-log regressions (logit Y vs. log X) to express the standard curve (13-18). Usual protocols lead to a region of linear response ranging from approximately 10-90% bound labeled MT (and approximately 50-15000 pg unlabeled MT); the extensions at high and low concentrations of unlabeled MT exhibit slopes about 1/4 that of the central region, which typically has a slope near 1 (negative). Use of the extended region, particularly that involving 0-50 pg unlabeled MT, is concomitant with increasingly large uncertainties associated with the increasing variance of measurements as the fraction of bound labeled MT approaches unity. However, with care in the experimental procedures one can develop standard curves over this region permitting the quantitation of samples with MT concentrations as low as 0.01 ng/mL. Typically, measurements at 2ng/mL carry an SEM (standard error of the mean) of 5-10% of the mean, and this increases gradually

over the next two decreasing orders of magnitude in MT concentrations to about 100% of the mean at 0.01 ng/mL; this sets the practical lower limit of quantitation at about 0.02 ng/mL.

Examples of some recent determinations of MT concentrations in normal human serum are shown in Table 1. The results are from an experiment preliminary to the quantitation of sera from humans suffering disorders associated with faulty metal metabolism and thus suspected of being MT-related. As effort was therefore made to extend quantitation to include concentrations of MT at the absolute limit of detection. For the preliminary experiment, serum was collected from 17 carefully selected volunteers (Syracuse University students, young, healthy, nonsmokers), and the MT content was determined in each such sample. The distribution, with a median value at approximately 0.1 ng/mL, may serve as a guide in the continued development of kinetic theories of metal and MT metabolism in the body (19,20).

As a test of reproducibility of results at the limit of detection, separately prepared aliquots of the samples with MT concentrations less than 0.1 ng/mL were then mixed (in varying amounts) for the development of a standard curve by using 100  $\mu$ L aliquots. The previously measured MT contents of the original aliquots were used to calculate the expected mean value for the mix of the second set. The mean value calculated for the mix was 0.043(0.017) ng/mL; the measured value for the mix of the second set was 0.078(0.037) ng/mL. The two mean values are not statistically distinguishable; note that two separate regressions are involved here, the second slightly less accurate than the first.

These results support the thesis that careful handling of samples, especially the technique of

**Table 1. Metallothionein (MT) concentrations in normal human serum.<sup>a</sup>**

MT range, ng/mL <sup>b</sup>	Number
0.01 (0.01) - 0.10 (0.03)	8
0.10 (0.03) - 0.50 (0.12)	5
0.50 (0.12) - 1.00 (0.18)	4

<sup>a</sup>Samples from young, healthy, nonsmokers at Syracuse University.

<sup>b</sup>Standard error of typical mean values at the indicated concentrations in parentheses. Quantitation used two standard curves relating logit of fraction bound labeled MT to log (Q) of concentration (in pg) of unlabeled MT. These intersected at Q = 1.6317, corresponding to 42.83 pg unlabeled MT. The two slopes (negative) in this system of units were 0.205 and 0.701, respectively. The correlation coefficient (negative) decreased from 0.987 (high concentration region) to 0.931 (low concentration region).

initially preparing separate aliquots of a given sample prior to freezing and storage, minimizing oxidizing atmospheres and handling times at elevated temperatures, and use subsequently of a given aliquot but once, permit experimental operations which lead to results in satisfactory agreement with predictions or with previously determined results, even when working at the absolute limit of practical detection of MT.

A similar set of experiments on MT content in normal urines from the same group of volunteers mentioned above led to a spectrum of concentrations (group A, Table 2) which we have found to be typical of normal urines. We include data (group B, Table 2) from a determination of MT content in control urines submitted in conjunction with an analysis of MT content in body fluids of patients with neurological disorders and another set of data (group C, Table 2) from a determination of MT content in control urines submitted in conjunction with an analysis of MT content in body fluids of workers exposed to cadmium (5). The three determinations were performed at various times and under slightly different protocols; the MT ranges listed and the associated standard errors of typical measurements are therefore not identical. Additionally, the control urines were measured concomitantly with samples expected to have MT contents in excess of 10 ng/mL and the standard curves developed for quantitation had a practical lower limit of detection of about 1 ng/mL. The values of these three sets for those samples listed in Table 2 with MT content less than 10 ng/mL lead to a range of mean values, each carrying an uncertainty of about 30%, from 2.8 to 5.1 ng/mL.

**Table 2. Metallothionein (MT) concentrations in normal human urine.<sup>a</sup>**

Group A		Group B		Group C	
MT range, ng/mL	Number	MT range, ng/mL	Number	MT range, ng/mL	Number
< 2	2	< 1.6	2	< 1	6
2-5	3	1.6-2.6	9	1-2	5
5-10	4	2.6-5	8	2-5	3
> 10	1 <sup>b</sup>	5-10	3	> 10	4 <sup>c</sup>

<sup>a</sup>Samples in group A from young, healthy, nonsmokers at Syracuse University. Samples in group B from controls submitted as part of an experiment on MT content in patients with spina bifida (collaboration with A. Zimmerman, Univ. of Connecticut). Samples in group C from controls submitted as part of an experiment on workers exposed to cadmium (5). Standard errors of typical means range from 50% at the lowest reported concentrations to 15% at 10 ng/mL.

<sup>b</sup>22 ng/mL.

<sup>c</sup>One value of 130 ng/mL.

## Summary Comments

The characteristic range of concentrations of MT in normal sera is from undetectable to about 1 ng/mL; concentrations above 2 ng/mL are considered abnormal. The characteristic range for normal urines is from less than 1 ng/mL to about 10 ng/mL; concentrations above 10 ng/mL are considered abnormal. In normal subjects, the ratio of urine MT/serum MT in a given case typically ranges from 10 to 50. In passing, we comment that cytosols (kidney, liver) have MT concentrations which are commonly orders of magnitude greater than typical serum concentrations. Measurements of samples of cytosols are readily made in the central region of the standard curve where standard errors of the mean of measurements are about 5% of the mean. In that regard, if greater accuracy is desired, such is attainable by relatively simple modifications of the protocols for developing the standard curve.

## Progress in Developing an ELISA

We have been working on the development of an ELISA (21-23) or enzyme-linked immunosorbent assay as a complement to the RIA. The protocol involves use of an antigen (Ag) fixed to a microtiter plate (fixed Ag), antigen subsequently added in solution (free Ag) to compete with the fixed Ag for a primary antibody (Ab1), a secondary antibody (Ab2) conjugated to an enzyme, the conjugate binding with the complex of fixed Ag-Ab1 after disposing of the complex of free Ag-Ab1, and a substrate which binds to the enzyme in the conjugate. We have been using the enzyme, alkaline phosphatase, conjugated to protein A. The substrate may be selected to permit detection of the final complex by colorimetric or fluorimetric means. We have focused on the colorigenic *p*-nitrophenyl phosphate and the fluorogenic 4-methylumbelliferyl phosphate in these two detection methods.

At this writing the practical detection and quantitation limits for MT are about 50-5000 pg for fluorimetric methods and about 500-50000 pg for colorimetric methods (J. Linton, unpublished results). Errors have been about twice those typical of the RIA.

## Antigenic Determinants and Secondary Structure of MT General

In the development of an RIA for the detection and quantitation of a particular molecule (antigen; Ag), there are two principal phases. The first

is an *in vivo* phase in which the Ag stimulates an immune response leading to the production of other molecules (antibodies; Ab) specific for certain sites (determinants) on the Ag surface, these sites commonly involving four to six residues and to varying degrees influenced by the topography of the surface near the site (24–26). This response involves amino acid sequence, the presence of charged residues, surface conformation, exposure to solvent, and the character of adjacent regions. The specificity of the immune reaction is then reflective of the characteristics of primary structure (sequence), secondary structure (helix, beta chain, reverse turn, random coil, intra-chain interactions) and tertiary structure (inter-chain interactions). In the second, *in vitro*, phase, the produced Ab molecule is used as a sensitive probe of surface structure of other molecules, both similar and dissimilar to the initial Ag of the *in vivo* phase. The *in vitro* physical-chemical reaction is again reflective of the same parameters of structure that characterized the *in vivo* reactions. In conjunction with predictions of relative hydrophilicity (27–29) and of probable protein structure from sequence analysis (30–34), one can interpret the results of competitive binding RIAs to indicate not only specific variations in sequence between Ag molecules and variations in surface structure in and near determinants, but also to indicate regions where the predictions of surface structure (helices, beta chains, reverse turns) based on sequence and hydrophilicity are either confirmed or contradicted. Coupled to previously available information from spectroscopic and other experiments, the assay results can be used to support or amend existing concepts of secondary structure, and to a certain extent tertiary structure, and to suggest details of surface exposure and core folding (35–37).

Our analysis of the structure of MT is based on the protocols developed by Chou and Fasman (32,33) and influenced by the hydrophilicity scale developed by Hopp and Woods (28), the latter in turn based on the analysis of relative hydrophobicity of amino acids by Nozaki and Tanford (27) and subsequent modifications by Levitt (30,31). The Chou/Fasman protocols are based on the study of experimentally determined conformations in 29 proteins, primarily globular and averaging about 150 residues each, including histidine and the aromatics. These protocols replace an earlier set based on analysis of 15 proteins; the earlier set was the basis of a calculation (38) which concluded that helices were forbidden in human MT-2 and that beta chains were only marginally possible. The improved protocols mod-

ify significantly the earlier set and contain additional boundary conditions to permit more accurate evaluation of the potential for a given conformation to exist. In passing, we remark a recent analysis (39) of the secondary structure of human MT-2 using abbreviated Chou/Fasman protocols (40) for determining candidate helices and beta chains. However, the use of the numerous boundary conditions in the complete protocols modifies the initial predictions regarding candidate conformations and additionally we are interested in comparisons of potential antigenic sites in the MTs of common use in the RIA (human, rat, rabbit). An analysis of secondary structure in the various isoforms of these MTs as derived from the complete predictive theory is therefore requisite.

In applying these protocols to vertebrate MTs, it should be noted that MT is smaller than most of the proteins used to derive the protocols, has an unusual amino acid composition, contains typically 16 rather than 20 amino acids, and is elliptical rather than globular (41–43). Further, it exists in the native state as two semi-independent clusters (44–47) identified as cluster B (residues 1–29) and cluster A (residues 30–61). Secondary structure will also be influenced by the fact that the native protein binds 6–7 divalent metal atoms at neutral pH (42,43) although recent potentiometric experiments indicate that the number of cadmium atoms in a cadmium-MT bound *strongly* at pH 7 is only 5 (A. Avdeef, A. Zelazowski, and J. S. Garvey, unpublished observations). The apoprotein has been numerous reported as essentially random coil (38,48,49); since the RIA demonstrates cross-reactivity between the native protein and the apoprotein (1–3), this suggests that the apoprotein is conformable to the native shape by the binding antibody, at least in respect to antigenic determinants. There is no reported firm experimental evidence for alpha helices or beta chains in the native protein and a contribution of less than 5% to MT secondary structure has been estimated from various spectroscopic studies for these conformations (50).

### Correlation of Predicted Secondary Structure of MT with Experimentally Derived Sites of Antigenic Determinants

The MTs analyzed by using the Chou/Fasman protocols in association with the Hopp/Woods hydrophilicity profiles included the most commonly reported isoforms of human, rabbit, rat and

	10	20	30	40	50	60
HUMAN MT-2	Ac MDPNCSCAAGDSCTCAGSCCKCKECKCTSCCKKSCCSCCPVGC AKCAQGCICKGASDKCSCCA					
HUMAN MT-1		G				
RABBIT MT-B		D G	T		S	
RAT MT-2		T D G	S		( S	Q )
RAT MT-1		S T G	S T G	N	( S	V A T )
EQUINE MT-1A		P T G		R	G R G	V
EQUINE MT-1B		V E		Q R A		V

FIGURE 1. Amino acid sequences of vertebrate metallothionein isoforms. Sequences shown are the most common variants of each isoform. Substitutions with respect to human MT-2 are indicated. Sources: human MTs 1 and 2 (51-53); rabbit MT-B (54); rat MTs 1 and 2 (55,56); equine MTs 1A and 1B (53,57,58). Mouse MTs I and II (59,60) are used to complete the sequences of rat MTs 1 and 2, respectively (in parentheses). The substitutions indicated for the various MT sequences with respect to that of human MT-2 are the obligate ones only. Human MT-1 variants may involve the appearance of P in position 9, V in position 10 or 12, the substitution of A for T or vice versa in positions 9, 14 and 53, or E substituted for D in position 55. Rabbit MT-B may substitute A for E in position 23.

equine MTs: human 1 and 2 (51-53), rabbit B (54), rat 1 and 2 (55,56) and equine 1A and 1B (53,57,58). Mouse MTs I and II (59,60) were used to complete the incomplete sequences of rat MTs. Figure 1 exhibits the sequences of these various MT isoforms. Note that because of numerous allowed substitutions there exist variants of these isoforms (43,61). The substitutions indicated for the various MT sequences with respect to that of human MT-2 are the obligate ones only (see Fig. 1). All the isoforms have 61 residues of which 20 are cysteine (C), approximately 12 are charged residues [aspartic acid (D), glutamic acid (E), lysine (K) and arginine (R)], approximately 8 are serine (S), another 10 are either alanine (A) or threonine (T), approximately 5 are glycine (G), and the remnant includes methionine (M), proline (P), asparagine (N), glutamine (Q), leucine (L), isoleucine (I) and valine (V). The aromatics are absent and histidine (H) has been found only in chicken MT (61). The amino terminus always has an acetylated methionine followed by -DPNCSC- through residue 7.

The antigenic determinants of vertebrate MT have been identified (11) as localized in the segment -MDPNC- (residues 1-5) and the segment of residues 20-25 (-KCKECK- in human MT). Residues 30-61 (cluster A) exhibited trivial reactivity. The experiment involved using anti-rat MT antibody produced in rabbits. Rat MT cross-reacts with human MT and equine MT with only minor changes in affinity, implying that the same or but slightly altered determinants are involved in the separate reactions. In this regard, although the slopes of standard curves for rat, human, and equine MTs are statistically indistinguishable, those of equine MTs are typically shifted slightly in the direction of decreased reactivity (2,12), and this may reflect the modification of the determi-

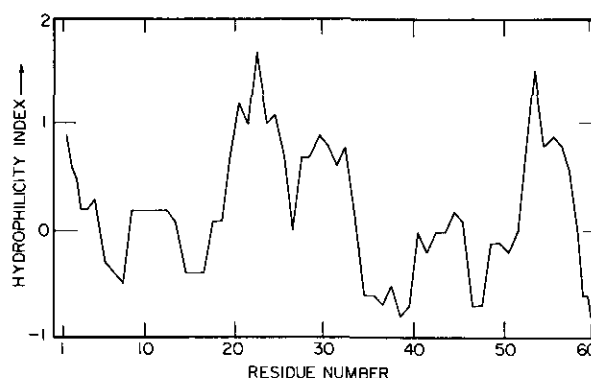


FIGURE 2. Hydrophilicity profile of human metallothionein isoform 2. Hydrophilicity indices (kcal/mole free energy change as solvent changes from water to an organic solvent) are indicated for sequential hexapeptides according to the protocol of Hopp and Woods (28). Values are plotted in the center of the hexapeptide sequence. The Hopp/Woods hydrophilicity scale derives from the amino acid solubility studies of Nozaki and Tanford (27) as subsequently modified by Levitt (30,31) and then again modified after empirical observations relating hydrophilicity and antigenicity. The terminal regions are completed with values for penta-, tetra-, tri- and di-peptides. The Hopp/Woods hydrophilicity indices are as follows for the amino acids in MT: 3(D, E, K, R); 0.3(S); 0.2(N, Q); 0(G, P); -0.4(T); -0.5(A); -1(C); -1.3(M); -1.5(V); -1.8(I, L).

nant in the region of residues 20-25 by the substitution of R for K in position 25 and/or the substitution of Q for E in position 23.

Figure 2 exhibits the hydrophilicity profile of human MT-2 according to the protocols of Hopp and Woods (28); the profiles of the isoforms are similar, the principal differences being in the magnitudes attained at the relative maxima. The protocols identify the hexapeptide (or its immediate neighborhood) with the maximum average hydrophilicity index as an antigenic determi-

nant; less certain identification is assigned hexapeptides of lesser hydrophilicity. For the vertebrate MTs studied, relative maxima occur in the region of residues 21–25, 29–33 and 52–58, with lesser relative maxima in the region of residues 9–13 and 42–46, although in the case of equine MT-1A and rat MT-1 the first mentioned region remains hydrophobic and the second mentioned region is minimally hydrophilic in all isoforms studied. The extended regions of residues 5–17 and 34–52 are either hydrophobic or minimally hydrophilic; the carboxyl terminal residues exhibit significant hydrophobicity.

Figure 3 exhibits the reverse turn profile of human MT-2 as derived from the Chou/Fasman protocols, and also exhibits the segments which qualify as potential candidates for helical or beta chain conformation on the basis of their conformational parameters (see Fig. 3); the latter two classes particularly require additional analysis (application of boundary conditions) before a prediction of their probable existence can be made. The predictive protocols are based on the frequencies of appearance of the 20 common amino acids in helices, beta chains, and reverse turns in the 4741 residues of 29 proteins. These frequencies are enumerated for each position in such conformations. A valuable boundary condition involves the sextet of residues at the amino and carboxyl terminals of helices and beta chains (i.e., the three residues preceding the amino terminal and the first three amino terminal residues, the last three residues at the carboxyl terminal and the first three residues following that terminal). The continued product of the site frequencies of the sextet at either terminal of the conformation represents a probability of formation which can be compared to the average value for that sextet and provides a guide in deciding on the probable occurrence of that conformation. A similar set exists for the tetrapeptides preceding and following reverse turns (we comment that we have not found this latter set of site frequencies to be as useful as the sets for helices and beta chains). Additional protocols involve the frequency of appearance of certain dipeptides and tripeptides in helices, beta chains and reverse turns. Each amino acid is then characterized with respect to helix formation and beta chain formation as a former (strong, moderate, weak), indifferent, or breaker (moderate, strong). A given sequence, influenced by residues adjacent to it, will have potential for forming a particular conformation if certain empirically derived conditions are met. Helices involve six or more residues, beta chains three or more residues, reverse turns four resi-

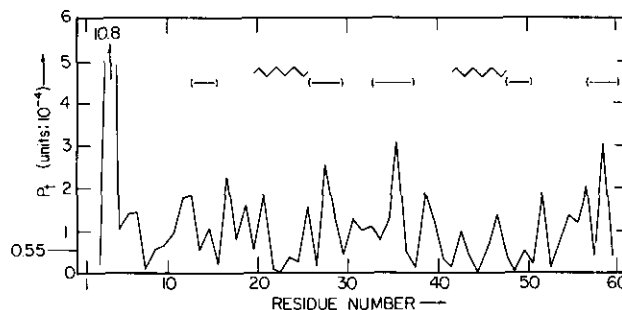


FIGURE 3. Reverse turn (beta bend) profile of human metallothionein isoform 2: (—) helices; (—) beta chains.

Probabilities  $p_i$  for sequential tetrapeptides to form a reverse turn according to the protocols of Chou and Fasman (32,33) are plotted, with values indicated in the center of the referenced tetrapeptide. The protocols are based on analysis of 29 proteins (4741 residues) which leads to the assignment of a frequency of appearance of a given amino acid in a specific position (1,2,3 or 4) in a reverse turn (similar frequencies are developed for helices and beta chains). The continued product of these site frequencies for a specific set of four residues represents the probability that the set will form a reverse turn. The average value is  $0.55 \times 10^{-4}$ . Values about twice the average value indicate a significant potential for such a conformation. Also indicated are the segments which satisfy the primary conditions of the protocols to qualify as candidates for formation of helices or beta chains. The measure used is the conformational parameter of the sequence; this is the average value of the conformational parameters of the member residues. In the analysis of the 29 proteins, each amino acid has an observed frequency of appearance in a particular conformation; this value divided by the frequency of appearance of all residues in that conformation yields a number (conformational parameter; typically between 0 and 3) which is a member of a normalized set of 20 members. To be predicted as a stable conformation the indicated helices and beta chains must additionally satisfy certain boundary conditions and secondary conditions regarding the appearance of various dipeptides and tripeptides and the relative magnitude of reverse turn potential involving the same residues. These conditions compromise the potential of both helices and at least three of the candidate beta chains and make their stable existence unlikely. The known structure of native MT (cluster formation) compromises the potential of the other two beta chain candidates.

dues. A given sequence or region may exhibit potential for forming more than one conformation and various boundary conditions are then applied to resolve the ambiguity. The most favored residues for appearance in a helix (strong formers) are E, M, A, L; moderately strong formers are K, Q, I, V; strong breakers are P and G. The most favored residues for forming a beta chain are V, I; moderately strong formers are L, C, T, Q, M; strong breakers are P, D, E. Residues in MT with the largest values for frequency of appearance in the sequential four positions of a reverse turn are: (1) N, C, D, S; (2) P, then S, K, D, T, R; (3) N, G, D, then S, C; (4) G, C, T, then S. For appearance in a

reverse turn without regard to position the most prominent residues are: N, G, P, D, S, then C, K. The residue with the largest frequency of appearance in any position of a reverse turn is P in position 2.

The reverse turn profile (Fig. 3) indicates that almost half of the sequential tetrapeptides have probabilities of formation which are in excess of the average value by a factor of two or more and as a class they dominate the complete conformational profile. The tetrapeptide common to all the isoforms studied with the highest potential to form a reverse turn is -DPNC- (residues 2-5), with a probability about 20 times the average value of  $0.55 \times 10^{-4}$ . This tetrapeptide is in a hydrophilic region and would be predicted to be a candidate for an antigenic determinant. The sequence -MDPNC- (residues 1-5) has been identified as a determinant and in this case the experimental findings support the theoretical prediction. We also mention that residues 37-40 in equine MT-1A (-CPGG-) and rabbit MT-B(-CPSG-) have high reverse turn probabilities ( $12.9 \times 10^{-4}$  and  $8.2 \times 10^{-4}$ ); the region, however, is hydrophobic. Other tetrapeptides in human MT-2 of high turn potential include the following in the principal hydrophilic regions: residues 19-22 (-CKCK-), 55-58 (-DKCS-), 24-27 (-CKCT-) and 53-56 (-ASDK-). Note that the tetrapeptide -CKEC- (residues 21-24) qualifies as a reverse turn with high potential (three times the average value) on the basis of site frequencies, but it is eliminated by the appearance of the dipeptide, -KE-, which does not occur in reverse turns. Numerous candidates appear in hydrophobic or minimally hydrophilic regions and would not be predicted to appear in antigenic determinants; such include the following of high turn potential: residues 34-37 (-CSCC-), 57-60 (-CSCC-), 26-29 (-CTSC-), 15-18 (-CAGS-), 37-40 (-CPVG-), 11-14 (-DSCT-), 50-53 (-CKGA-), 45-48 (-AQGC-), 41-44 (-CAKC-), 17-20 (-GSCK-), 33-36 (-CCSC-), 31-34 (-KSCC-) and 38-41 (-PVGC-). Substitutions in the other vertebrate isoforms modify the magnitude of the separate probabilities in the remarked regions (*vide infra*).

The regions of highest potential for forming a helix in human and rabbit MTs are those of residues 20-25 (-KCKECK-) and of residues 42-47 (-AKCAQG-). The first mentioned region remains a candidate, even after the substitutions indicated in Figure 1, in all the isoforms studied. The second region is not a candidate in the other MTs (rat and equine). Mouse MTs, used as a pattern for rat MTs in cluster A, also have a helix

candidate in the region of residues 51-56 (-KGAADK- or -KQASDK-). Note that the region 20-25 is prominently hydrophilic, and the region 42-47 is hydrophobic or minimally hydrophilic; the region 51-56 is hydrophilic. The sequence -KCKECK- satisfies the primary conditions for possible helix formation, but it has certain unfavorable aspects. The dipeptide, -KE- is unfavored for appearance in a helix and the sequence fails to satisfy the boundary condition mentioned earlier regarding the sextet of residues at either terminal of the helix. The continued product of the individual site frequencies for the sextet of amino acids in the three positions preceding the amino terminal of the candidate helix and the first three positions at that terminal is less than  $\frac{1}{5}$  the average value for helix initiation ( $0.67 \times 10^{-9}$ ). Coupled to the disfavored dipeptide, -KE-, this implies that the sequence will not form a stable helix. The tetrapeptides -CKCK- (residues 19-22) and -CKCT- (residues 24-27) have reverse turn probabilities three times the average value. For human MTs and rabbit MT-B we conclude that the antigenic determinant in the region of residues 20-25 involves one or both of the conformation(s) of the mentioned reverse turns and one or more of the charged residues in that sequence. The various substitutions in the other isoforms do not change the unfavorable character for helix formation of the amino terminal sextet. In rat MT-1 the substitution of G for K in residue 20 leads to a probability for helix initiation  $\frac{1}{3}$  the average value, while having minimal observed effect on the antigenicity of the region. The substitutions of Q for E in position 23 (as in equine MT-1B and rat MT-2) or N for E in that position (as in rat MT-1) or R for K in position 25 (as in equine MTs) slightly reduces but does not eliminate helix potential. The various isoforms in this residue region also exhibit high reverse turn probabilities for residues 19-22 and 24-27 (-CGCK-, -CRCT-, and -CRCA- have reverse turn probabilities two to three times the average value). In sum, the candidacy of residues 20-25 for helix formation appears significantly compromised by the boundary conditions and the higher potential of the region to support reverse turns. In this regard, reverse turns may terminate helices or beta chains, one residue a member also of the joining conformation. The Chou/Fasman protocols count as reverse turns only those with no more than the initial or final member of the turn common to both conformations; they do not include those types of reverse turns which may be considered part of a helix. The competing potentials we em-

phasize then are first those of tetrapeptides common to both conformations, then those with three or two members in common. With the above considerations in mind, and in view of the observed cross-reactivity of these vertebrate isoforms, one concludes that the antigenicity of the region is controlled by the conformational contributions of the mentioned reverse turns and the two charged residues in positions 22 and 25 (K,K in human, rabbit and rat MTs; presumably K,R in equine MTs).

The sequence of residues 42–47 (–AKCAQG–) in human MTs and rabbit MT–B is a helix candidate based on conformational parameter but the sextet of residues at the amino terminal is unfavorable for helix initiation (probability about  $\frac{1}{3}$  the average value). Residues 41–44 (–CAKC–) and 45–48 (–AQGC–) have high turn probability (about two and three times the average value). The substitutions in rat MTs and equine MT–1A in this region eliminate the sequence as a helix candidate; in the latter case residues 44–47 (–CAGG–) have a very high turn probability (about six times the average value), and in the case of the rat MTs the substitution of S for A in residue 42 produces the tetrad –CSKC– which has four times the average probability to form a reverse turn. The hydrophobicity or minimal hydrophilicity of the region indicates that it will not support a determinant, whether the conformation is helix or turn. The unfavorable boundary conditions for helix formation indicate that this region does not support a stable helix; however, it may be characterized by one or more reverse turns in all the isoforms studied. In passing, we note that the residues 48–50 (–CIC– or –CVC–) in all isoforms have high beta chain potential, and in those isoforms with –QG– in positions 46–47 the pentapeptide –QGCIC– (or –QGCVC–) has high beta chain potential and this may compromise the formation of those reverse turn candidates which include –QG–.

As noted, mouse MTs exhibit a potential helix candidate in the theoretically hydrophilic region of residues 51–56; in either case (–KGAADK– or –KQASDK–) the amino terminal sextet is very unfavorable to helix initiation (probabilities less than  $\frac{1}{20}$  the average value). Extensions to include residues 43–57 fail to yield a satisfactory conformational parameter (too few strong formers). Additionally, there is competition in the latter case with the beta chain candidates mentioned above (residues 48–50 or 46–50), which have beta chain potentials exceeding the helix potentials in that segment. Residues 50–53 (–CKGA–) and 55–58 (–DKCT–) in mouse MT–I

have reverse turn probabilities more than twice the average value; and in mouse MT–II reverse turn probability is high for residues 53–56, 54–57, and 55–58 (–ASDK–, –SDKC–, and –DKCS–; two, three and four times the average value, respectively). Assuming these residues characterize rat MTs, the region is most probably one of one or more reverse turns; the failure of the region to exhibit significant antigenicity presumably reflects suppression of the conformations by the chelation process in native MT, even though the sequence –DK– is favorable in reverse turns. The hydrophobicity of the adjoining regions may also act to suppress exposure and conformational potentials. Note also that tryptic digestion cleaves the –KC– bonds in cluster B but fails to do so in cluster A (46), again implying a region less exposed to solvent than theory would indicate.

Our discussion involving the potential conformations in the isoforms of MTs other than human MT–2 has focused on the obligate sequences indicated in Figure 1. The substitutions noted in the legend of Figure 1 involving human MT–1 and the residue region 9–14 tend to reduce the hydrophilicity index of the region and also the probability that the region will exhibit significant antigenicity; the substitutions involving residues 53 and 55 have a slight influence on helix potential (residues 51–56) but the sextet of residues at the amino terminal of that candidate remains very unfavorable for helix initiation (less than  $\frac{1}{20}$  the average value for that sextet). The substitution of A for E in position 23 in rabbit MT–B slightly improves the helix potential of the sequence 20–25 while significantly improving the reverse turn potential of all tetrads involving positions 22 and 23; the hydrophilicity index of the hexapeptide (residues 20–25) is reduced from 1.7 to 1.1, still a relative maxima and a candidate antigenic site, its character dominated by reverse turns and the two lysines. In passing we comment that mouse MT–I substitutes A for K (residue 20) and D for E (residue 23) in the sequence 20–25. The dipeptide –KD– is favorable to helix formation (and to reverse turn formation) as is the sextet at the carboxyl terminal; however, the sextet at the amino terminal has only half the average probability for initiating a helix and reverse turn probability remains unchanged for residues 24–27, only slightly reduced for residues 19–22 (still more than twice the average value) and increases for residues 21–25. We conclude that this isoform will exhibit a conformation and antigenicity similar to that of the previously mentioned isoforms. In this regard, certain metal-binding proteins from mouse ascites cells



(provided by David Petering, University of Wisconsin) have been shown to cross-react in RIAs with a rat liver (Cd, Zn)-MT and have been tentatively identified as MTs (unpublished observations).

The failure of the theoretically hydrophilic region joining the two clusters, residues 27–33, to exhibit antigenicity is perhaps a consequence of the sequestering of the region between the two clusters. The region has no helix potential but has a series of candidate reverse turns with probabilities of formation between two and five times the average value, and the dipeptide –KK– is favorable for turn formation.

Antigenicity is not expected to involve the hydrophobic regions of the candidate beta chains. We may note that of the five candidates in Figure 3, three have reverse turn potentials which dominate the beta chain potentials. These are residues 34–37, (–CSCC–) or the extension to 33–37 (–CCSCC–); residues 26–29 (–CTSC–); residues 57–60 (–CSCC–, although a possible exception is the sequence in mouse MT-I, –CTCC–). Two segments, residues 13–15 (–CTC–) and 48–50 (–CIC– or –CVC– in some isoforms), compete favorably with reverse turn and helix potentials, but they exist in separate clusters in native MT. It is concluded that coupling of any two segments of the five is improbable and that beta structure in native MT is at best unstable.

With respect to the previously remarked recent analysis (39) of the secondary structure of human MT-2 using only the conformational parameters of the abbreviated protocols (40) for predicting candidates for helical or beta chain conformation, the predictions are similar, although not identical, to our own. That analysis focuses, as do we, on the high potential of the total sequence to support reverse turns. In that regard, the recently modified sequence (53) at the carboxyl terminal (residues 57–60) changes –CCSC– to –CSCC– which significantly enhances the reverse turn potential of that tetrapeptide (see Fig. 3). The helix candidate listed in the referenced analysis as involving residues 41–46 is eliminated on applying the boundary conditions regarding the sextet of residues at the carboxyl terminal of that candidate. As we have noted, even the permitted candidate in that region (residues 42–47) has an unfavorable sextet of residues at the amino terminal for initiating a helix. However, these differences in analyses are minor with respect to the correlation of structure with antigenicity, residues 57–60 and 42–47 appearing in hydrophobic or minimally hydrophilic regions and exhibiting insignificant antigenicity.

## Summary Comments

As noted earlier, the conclusions of others (38,50) regarding the existence of either helices or beta chains in vertebrate MT isoforms have been negative or at best unfavorable. The predictions of secondary structure from our analysis are compatible with those conclusions, although they do not absolutely rule out the possible fluctuating existence of those conformations. The results of the experiment on the determinants of vertebrate MT, identifying –MDPNC– as a principal antigenic site, rather firmly support the theoretical prediction of a prominent exposed reverse turn (–DPNC–) at the amino terminal. And the results suggest that the determinant in the region of residues 20–25 (–KCKECK– in human MT) owes its character to reverse turns (residues 19–22 and/or 24–27) involving exposed and charged residues. The results also suggest that the joining region of clusters A and B may be sequestered between the two clusters and that the theoretical hydrophilicity and possible antigenicity of the region is consequently suppressed. Similarly, the results may be interpreted to suggest that the theoretical hydrophilicity and potential antigenicity of segments near the carboxyl terminal of cluster A may be suppressed in the native protein as a consequence of the chelation process, with potential antigenicity also compromised by small structural fluctuations reflecting competing conformational potentials.

*Note added in press:* A recent analysis of the sequence of rat MTs by D. R. Winge (unpublished) indicates the following substitutions in the sequences listed in the text. Rat MT-1: S for T in position 17; S for A in position 54. Rat MT-2: A for S in position 42; S for A in position 45, E for Q in position 52. In all cases these variations, although mildly influencing conformational potentials, leave unchanged the conclusions of the text regarding probable conformations in the affected regions. In MT-1 regional turn potentials increase slightly and the beta chain potential in the region of residue 17 is slightly decreased. In MT-2 turn potentials in the affected regions vary slightly, the beta chain potential in the region of residue 45 remains high, and the sequence 51–56 remains unfavorable for helix initiation with residues 53–57 still predicted to be a turn. The presence of E in position 52 does increase the hydrophilicity index of those hexapeptides of which it is a member, the maximum value (1.5) increasing to 2.0. Again, the failure of the region to exhibit significant antigenicity supports the conclusion of the text that the adjoining hydro-

phobic regions act to suppress in the native protein the exposure to solvent of the region of residues 51–56.

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